IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
(Case No. 06-439)

In the Application of:	)	
	)	
Nicolas Burdin et al.	)	
	)	**Examiner: Shawquia Young**
Serial No.: 10/596,432	)	
	)	**Group Art Unit: 1626**
Filing Date: June 13, 2006	)	
	)	**Confirmation No.: 6546**
For: Immunostimulant Composition Comprising At Least One Toll-Like Receptor 7 or Toll-Like Receptor 8 Agonist And A Toll-Like Receptor 4 Agonist	)	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF NICOLAS BURDIN, PhD.

I, Nicolas Burdin, PhD., in support of the above-identified patent application, do aver and state as follows:

1. I received a PhD. in Immunology from the ScheringPlough Laboratory for Immunological Research, Claude Bernard University, Lyon I, France in 1996. I conducted post-doctoral research in immunology in the department of Microbiology and Immunology at the University of California at Los Angeles, and the Department of Developmental Immunology at La Jolla Institute for Allergy and Immunology at San Diego, California. Since 1999 I have worked at Aventis Pasteur and its successor, Sanofi Pasteur. My current title is Director of Discovery Research, France, for Sanofi Pasteur. A copy of my curriculum vitae is attached hereto as Exhibit A.

2. I am the same Nicolas Burdin who is the first-named inventor on the above-identified patent application.

3. This patent application is based on our surprising discovery that the combination of certain pairs of agonists in a single composition provides significantly superior results compared to what would have been expected based on the results achieved with each agonist used independently of the other. An important feature of the invention is the fact that the response observed is a potentiated response and more oriented towards the Th1 immune response.

4. This is even more surprising because it had been believed by researchers in this field that if two toll-like receptors were combined, not only would there be no synergistic effect, but there also would be a saturation phenomenon so that no additional effect of the second TLR would be observed. This is consistent with our findings, explained more fully below, that the combination of a TLR 2 agonist and a TLR 4 agonist did not provide any additional benefit.

5. The following is a detailed description of the procedures followed to assess a combination of TLR 2 and TLR 4 agonists against the results obtained with either of the two agonists used alone.

a. Preparation of a Stock Suspension of Agonists of the Toll-like 4 Receptor

Dipalmitoylphosphatidylcholine (DPPC) in powdered form was obtained from Avanti Polar Lipids (Alabaster, Ala.). The toll-like 4 receptor ER804057 in powdered form was obtained from Eisai, Inc., headquartered in Woodcliffe Lake, New Jersey.

273 μg of DPPC (0.372 μmol), supplemented with 150 μg of ER804057 (0.093 μmol), were dissolved in 760 μl of a chloroform/methanol 4:1 (vol/vol) mixture. The solution was dried in a round-bottomed glass flask with the aid of a rotary evaporator so as to leave a homogeneous lipid film on the walls of the round-bottomed flask. This film was further dried under a high vacuum in order to remove any trace of residual solvent, and then taken up in 3 ml of water at 60°C. The resulting liposomal suspension was homogenized by vortexing, followed by sonication in an ultrasound bath and then sequentially extruded with the aid of a

Lipex extruder thermostated at 50°C., in a passage across a polycarbonate membrane having a porosity of 0.8 μm, followed by a passage across a membrane having a porosity of 0.4 μm and finally a passage across a membrane having a porosity of 0.2 μm.
DPPC/ER804057 (4:1 mol/mol) liposomes suspension was thus obtained in water at 91 μg/ml of DPPC and 50 μg/ml of ER804057.

b. Preparation of a Stock Suspension of Agonists of the Toll-like 2 Receptor

The toll-like 2 receptor H8820, was obtained in powdered from Bachem, headquartered in Bubendorf, Switzerland.

69 μg of DPPC (0.094 μmol), supplemented with 390 μg of H8820 (0.375μmol), were dissolved in 528 μl of a chloroform/methanol 4:1 (vol/vol) mixture. The solution was dried in a round-bottomed glass flask with the aid of a rotary evaporator so as to leave a homogeneous lipid film on the walls of the round-bottomed flask. This film was further dried under a high vacuum in order to remove any trace of residual solvent, and then taken up in 3 ml of water at 60°C. The resulting liposomal suspension was homogenized by vortexing, followed by sonication in an ultrasound bath and then sequentially extruded with the aid of a Lipex extruder thermostated at 50° C., in a passage across a polycarbonate membrane having a porosity of 0.8 μm, followed by a passage across a membrane having a porosity of 0.4 μm and finally a passage across a membrane having a porosity of 0.2 μm.

H8820/DPPC (4:1 mol/mol) liposomes suspension was thus obtained in water at 23 μg/ml of DPPC and 130 μg/ml of H8820.

c. Preparation of Stock Suspensions of Agonists of the Toll-like 2 Receptor and Agonists of the Toll-like Receptor 4

150 μg of ER804057 (0.093μmol), supplemented with 390 μg of H8820 (0.375 μmol), were dissolved in 604 μl of a chloroform/methanol 4:1 (vol/vol)

mixture. The solution was dried in a round-bottomed glass flask with the aid of a rotary evaporator so as to leave a homogeneous lipid film on the walls of the round-bottomed flask. This film was further dried under a high vacuum in order to remove any trace of residual solvent, and then taken up in 3 ml of water at 60° C. The resulting liposomal suspension was homogenized by vortexing, followed by sonication in an ultrasound bath and then sequentially extruded with the aid of a Lipex extruder thermostated at 50°C., in a passage across a polycarbonate membrane having a porosity of 0.8 μm, followed by a passage across a membrane having a porosity of 0.4 μm and finally a passage across a membrane having a porosity of 0.2 μm.

ER804057/H8820 (1/4 mol/mol) liposomes suspension was thus obtained in water at 50 μg/ml of ER804057 and 130 μg/ml of H8820.

d. Preparation of the Vaccine Compositions

Vaccine compositions were prepared comprising, as vaccine antigen, a recombinant protein capable of being used in a vaccine against AIDS. This protein is the detoxified TAT III B protein which is obtained by expression in E. coli and purification by various chromatographic steps, followed by chemical inactivation, as is described in patent application WO99/33346, where it is identified under the term carboxymethylated Tat, and referred to hereinafter as "Tat."

The liposomal suspensions prepared according to sub-paragraphs (a)-(c) above each were mixed volume for volume (0.9 ml+0.9 ml) with a concentrated Tat solution at 200 μg/ml in 100 mM Tris buffer containing 200 mM NaCl, pH 7.4, in order to obtain the preparations (1.8 ml final) whose composition are indicated below and in which the quantities of antigens and of adjuvant are indicated per 200 μl dose:

1) Tat (20 μg)

2) Tat (20 μg)+ER804057/DPPC (5 μg/9.1 μg)

3) Tat (20 μg)+H-8820/DPPC (13 μg/2.3 μg).

4) Tat (20 μg)+ER804057/H-8820 (5 μg/13μg)

e. Immunization Test on Mice

Four groups of 6 female BALB/c mice 8 weeks old were each injected subcutaneously with one of the four compositions prepared in sub-paragraph (d) above at a dosage rate of 200 μl per mouse. The injections were performed on day zero and on day 21.

Blood samples were collected at the retro-orbital sinus at day 14 for assessing the primary response and at day 32 for the secondary response. The determination of the level of specific IgG1 and IgG2a was carried out by means of the standard ELISA tests.

To monitor cellular responses, the mice were sacrificed on day 37; their spleens were removed and the splenocytes were isolated. More particularly, spleens were collected after cervical dislocation of mice. The spleens were put individually into a collector filled with transport medium and pressed throughout a filter membrane to separate blood cells from the tissue. The suspensions were centrifuged slowly. Lysis of red blood cells was performed on the pellet with Gey's reagents. Splenocytes were then washed, numerated and conserved in medium at 37°C until use in cellular assay.

The results obtained for humoral responses are summarized in Table I below, where the IgG levels are expressed as arbitrary ELISA units (log10).

For each group of mice, the value indicated in the table is the mean geometric titer of the values obtained for each of the mice.

Table I

Vaccine Composition	IgG1 at day 14	IgG2a at day 14	IgG1 at day 32	IgG2a at day 32	IgG1/IgG2a ratios at day 32
Tat	1.897	1.000	4.343	2.436	176.2
Tat+ ER804057	2.598	2,820	5.101	4.838	3.5
Tat + H-8820	3.035	1.717	5.227	4.126	66.3
Tat + ER804057 +H-8820	3.364	2.475	5.350	4.621	15.9

Cell mediated immune responses elicited by vaccines in immunized mice were monitored by ELISA tests that measure the secretion of the IL5 cytokines or of γ Interferon in culture supernatants comprising splenocytes cultured in the presence or absence of recombinant TAT for 5 days. The results which are expressed in pg/ml are summarized in Table II below:

Table II

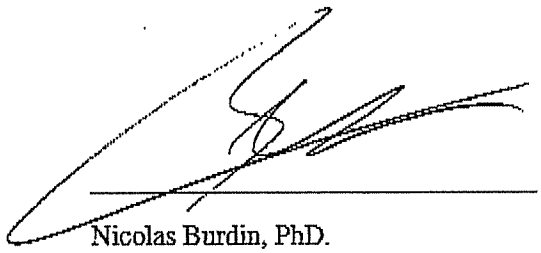
Vaccine Composition	IL-5	INF-γ	IL5/ INF-γ ratios
Tat	2893	7726	0.37
Tat + H-8820	727	2247	0.32
Tat + ER804057	220	3886	0.06
Tat + H-8820 + ER804057	469	5370	0.09

6. The data in Tables I and II indicate that while each of the TLR 2 and TLR 4 receptors when used alone exhibited an effect of the IgG levels (especially of IgG2a, hallmark of a Th1 orientation in mice) relative to the results obtained when no TLR agonist was used, the combination of the two receptors did not lead to improved results, and for some tests the results were actually lower than the results achieved when only one of the receptors was used. The same observation was made for IFNγ secretion (a cell-mediated hallmark of Th1 orientation). From these results, one skilled in the art of immunology would have expected that a combination of TLR 4 with either TLR 7 or TLR 8 agonists would not have led to improved results.

I hereby state that all statements made in this Declaration of my own knowledge are true, and all statements made on information and belief are believed to be true. I have been warned that willful false statements and the like are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon.

30/07/09

Date



Nicolas Burdin, PhD.